

**EVALUATION OF CIRCULATORY AND SALIVARY
LEVELS OF HEAT SHOCK PROTEIN 60
IN PERIODONTAL HEALTH AND DISEASE**

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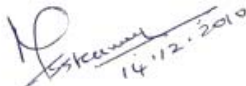


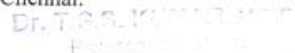
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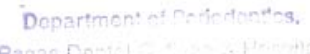
This is to certify that this dissertation titled "EVALUATION OF CIRCULATORY AND SALIVARY LEVELS OF HEAT SHOCK PROTEIN 60 IN PERIODONTAL HEALTH AND DISEASE" is a bonafide record of work done by **Dr. R.RAMYA NETHRAVATHY** under my guidance during the study period 2008-2011.


This dissertation is submitted to THE TAMILNADU DR.MGR MEDICAL UNIVERSITY in partial fulfillment for the Degree of **MASTER OF DENTAL SURGERY, BRANCH – II PERIODONTOLOGY**. It has not been submitted (partial or full) for the award of any other degree or diploma.


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LIST OF ABBREVIATIONS

CAL	-	Clinical Attachment Level
CD	-	Cluster Differentiation
Cpn	-	Chaperonin
CRP	-	C Reactive Protein
ELISA	-	Enzyme Linked Immuno Sorbent Assay
GCF	-	Gingival Crevicular Fluid
GroEL	-	Growth of Escherichia Coli Large
HSP	-	Heat Shock Protein
HumHSP	-	Human Heat Shock Protein
ICAM	-	InterCellular Adhesion Molecule
IFN	-	Interferon
Ig	-	Immunoglobulin
IL	-	Interleukin
LPS	-	Lipopolysaccharide
MHC	-	Major Histocompatibility Complex
MMP	-	Matrix Metalloproteinase

MycHSP	-	Mycobacterial Heat Shock Protein
NK	-	Natural Killer
PAMPs	-	Pathogen Associated Molecular Patterns
PBMC	-	Peripheral Blood Mononuclear Cells
PBS	-	Phosphate Buffered Saline
PPD	-	Periodontal Probing Depth
RA	-	Rheumatoid Arthritis
RANKL	-	Receptor Activator of Nuclear Factor – κ B Ligand
STAT	-	Signal Transduction and Activation of Transcription
T reg	-	Regulatory T Cell
TCR	-	T Cell Receptor
TGF	-	Transforming Growth Factor
Th	-	Helper T Cell
TLR	-	Toll Like Receptor
TNF	-	Tumor Necrosis Factor
VCAM	-	Vascular Cell Adhesion Molecule
WBC	-	White Blood Cell

Abstract:-**Background:**

Self antigens such as Heat shock protein 60 (HSP 60) have recently been implicated in the periodontal disease pathogenesis. There is scant evidence regarding HSP 60 in circulation and saliva following periodontal disease and its possible relation to systemic inflammation.

Materials and methods:

Forty five peripheral blood samples and saliva samples were collected from two groups of patients (periodontal health-Group A and periodontal disease- Group B). The serum, cell lysates, and saliva samples were used to detect and compare the HSP 60 levels in both the groups by Enzyme Linked Immuno Sorbent Assay (ELISA) technique. Statistical analysis was done using the student t test and Pearson's correlation.

Results:

Circulatory HSP 60 significantly increases in periodontal disease compared to health. There was a significant correlation between the total circulating HSP 60 but not cell lysate and serum levels in periodontal disease. There was no significant association between salivary HSP 60 and periodontal disease or systemic inflammation.

Conclusion:

Circulating HSP 60 levels seem to play role in the systemic inflammatory state produced by periodontal disease. Salivary HSP 60 may not be used as a surrogate to determine systemic inflammation.

Key words: HSP 60, Periodontitis, Peripheral blood, Cell lysates, ELISA.

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INTRODUCTION

Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or groups of specific microorganisms resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession or both.⁴⁴ Porphyromonas gingivalis, a gram negative anaerobic bacteria has been strongly implicated in the pathogenesis of chronic periodontitis, either singly or in combination with other red complex bacteria such as Tannerella forsythia, Treponema denticola.⁶⁵ The major antigenic determinants of these pathogens are Lipopolysaccharides, capsule, fimbriae, outer membranous protein; etc.²³ Although the inflammatory response in periodontal disease is evoked primarily in response to these exogenous antigens, structural similarities with host antigens may result in their activating the immune system through molecular mimicry.⁸¹

Heat shock proteins (HSP) are perhaps, the most extensively investigated self antigens involved in periodontal disease. These proteins are produced by cells in response to stress stimuli, including high temperature, mechanical stress, infection, surgical stress, oxidant and cytokine stimulation.⁴⁰ They participate in vital physiological processes in the cell such as folding, assembly, and translocation of polypeptides across membranes and are hence referred to as molecular chaperones. HSP 60 or Cpn 60 is a mitochondrial chaperonin that assists in folding linear amino acid chains into

their respective three-dimensional structure. Human HSP 60 (HumHSP 60) and their bacterial counterparts share more than 50% amino acid sequence homology, as a result of which the immune system may be inadvertently activated against these self antigens.¹⁴

Petit et al, *Ueki et al* have described a role for HSP 60 in periodontal disease. These authors have demonstrated an upregulation of HSP 60 expression in gingival tissues affected by periodontal disease. A potential role for these proteins in the etiopathogenic process has also been proposed based on the structural similarity between HSP 60 (GroEL) of *Porphyromonas gingivalis* and human gingival fibroblast. HSP 60 was shown to induce secretion of proinflammatory cytokines in professional antigen presenting cells.^{79,27} HSP 60 increases the activation of T cells in primary stimulation and thereby perpetuates the overall inflammatory process.⁷⁹

HSP 60 has been identified in serum even though they are typically regarded as intracellular components.⁵⁶ The source of this freely circulating HSP 60 is not fully understood and has hence, been the object of much speculation among some investigators.⁵⁷ Regardless of the source; circulating HSP 60 levels may assume clinical significance because it has been implicated in the etiopathogenesis of cardiovascular disease. HSP 60 has been reported to upregulate E-selectin, ICAM 1, VCAM 1 expression and interleukin 6 production in endothelial cells & thereby contribute to atheromatous plaque formation.³⁴

It is not yet known if periodontal disease contributes to this circulating HSP 60 although there is some evidence of a systemic spill over of several inflammatory mediators.²⁵ It is also not clear if circulating HSP 60 contributes to the systemic inflammation observed in periodontal disease, as evidenced by increased hs CRP levels.

Saliva has been used as a diagnostic tool to investigate periodontal disease activity as it is readily available and contains locally produced microbial and host response mediators.⁵⁴ Saliva can be collected noninvasively in large amounts, & with less patient discomfort, when compared to gingival crevicular fluid.³¹ Several inflammatory markers and host derived products in saliva such as IL-1, TNF α , MMPs, human neutrophil elastase, etc have been evaluated for use as markers for periodontal disease activity.^{39,70} However there is scant literature regarding the use of salivary HSP 60 as a biomarker.

In recent years saliva has been postulated to be capable of reflecting not only periodontal disease but also systemic diseases such as, Cardiovascular disease⁵⁴ Rheumatoid arthritis, Sjogrens syndrome.¹¹ There is insufficient evidence in literature regarding the reliability of using saliva as a surrogate for evaluating systemic inflammatory status and its mediators.²¹

The purpose of this study is to estimate circulatory and salivary levels of HSP 60 in periodontal disease and to assess whether they correlate with each other and with the systemic inflammatory status.

AIMS AND OBJECTIVES

The objectives of the present study are,

- 1) To estimate the circulatory levels of HSP 60 in periodontal health and disease.
- 2) To identify whether circulating HSP 60 levels correlate with hs CRP in periodontal disease.
- 3) To estimate the salivary levels of HSP 60 in periodontal health and disease.
- 4) To correlate salivary HSP 60 levels with circulatory HSP 60 and hs CRP.

REVIEW OF LITERATURE

Periodontal disease is characterised as a peripheral infection involving multiple species of gram negative organisms. The pathogenic species present in sub gingival biofilm release an array of virulence factors that can evade antibacterial host defence mechanisms and then cause damage to host tissue via immune or inflammatory interactions, which typically consist of neutrophils, monocytes/ macrophages, T cells and B cells.

*Socransky & Haffajee 1990*⁶⁵ proposed the presence of complexes of bacteria residing in dental plaque of which the red complex(*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) are thought to be closely associated with pathogenesis of periodontal disease.

These red complex bacteria are involved in the production of many virulence factors, such as capsule, Lipopolysaccharide, fimbriae, GroEL heat shock protein, outer membranous protein of gram negative bacteria. These antigens are believed to result in activation of host immune and inflammatory responses, which involves the generation of cytokines, recruitment of inflammatory cells, and the activation of osteoclasts.

It has recently been recognized, that many pathogens express virulence genes only when they are in their human or animal host. **Handfield and coworkers**²³ described an ingenious approach to distinguishing such

virulence factors. If a specific virulence factor were to play a role in the pathogenesis of infectious diseases, then that factor would be likely to elicit an antibody response in an individual with the disease which would not be observed in serum samples from subjects who did not have the disease. Using these techniques, there is increasing evidence regarding the role of self antigens in periodontal disease.

Role of self antigens in periodontal disease:-

In recent years it has been recognized that the immune response to self antigens may contribute to the disease process. High titres of anti collagen type I antibody have been identified in sera and collagen type specific T cell clones can be identified in infected gingival tissues in periodontitis.

Exogenous antigens may themselves help to elicit responses from host self antigens in the following way. An exogenous antigen may present with structural similarities with certain host antigens; thus, any antibody produced against this antigen (which mimics the self – antigens) bind to the host antigens and amplify the human response. The most striking form of molecular mimicry is observed in Group B-hemolytic streptococci, which stores antigens in humans, and is responsible for the cardiac manifestations of Rheumatic fever.

The immune system can fail in one of three ways.

1. Mistaken recognition of self antigens – autoimmunity
2. An ineffective immune response – immunodeficiency.
3. An overactive immune response – hypersensitivity.

An essential requisite for the immune system is the ‘Immunological Tolerance’, which is the ability of an individual to differentiate ‘**self**’ from ‘**non-self**’. This breakage leads to the Immune System mounting an effective and specific immune response against self determinants. The exact genesis of immunological tolerance is still elusive, but several theories have been proposed since the mid-twentieth century to explain its origin. There are three hypotheses in this regard,

- **Clonal deletion theory**, proposed by **Burnet**, according to which self – reactive lymphoid cells are destroyed during the development of the immune system in an individual.
- **Clonal Anergy theory**, proposed by **Noonoal**, in which self – reactive T or B cells become inactivated in the normal individual and cannot amplify the immune response.
- **Idiotypic Network theory**, proposed by **Jerne**, wherein a network of antibodies capable of neutralizing self-reactive antibodies exists naturally within the body.

Tolerance can also be differentiated into ‘central’ and ‘peripheral’ tolerance, on whether or not the above checking mechanisms operate in the central lymphoid organs (Thymus and Bone marrow) or the peripheral lymphoid organs (lymph node, spleen etc., where self-reactive B-cells may be destroyed). If these tolerance mechanisms are attenuated/reset; then the host antigens start acquiring a pathogenic character.

Molecular mimicry between bacterial and HumHSP may allow microorganisms to evade the host defenses. Immune responses to bacterial HSP may generate cross reacting immunity to self-HSP and precipitate damaging inflammatory responses

Three models have been proposed to link microbial infections to subsequent autoimmune reactions involving HSP. These models are based on (i) molecular mimicry between microbial HSP and HumHSP or constitutive proteins from the host, (ii) inflammation-induced exposure of cryptic cell epitopes that could be a target for immune reactions, and (iii) antigen persistence in infected sites leading to chronic immunological reactions.

Major periodontopathic bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Tannerella forsythia*, and *Campylobacter rectus* are reported to produce HSP homologous to *Escherichia coli* GroEL. Bacterial HSP 60, termed GroEL, are major antigenic determinants during infection, and since human and bacterial HSP 60 have a 55% homology, it is possible

that immune response to bacterial challenge may result in cross reactivity by targeting host cells expressing HumHSP 60.

Heat Shock Protein 60 and periodontal disease:-

Heat shock proteins (HSP) are grouped in families according to their molecular mass, and the human and bacterial cognates are very similar, sharing more than 50% sequence homology at the amino acid level.

In response to stress stimuli, including high temperature, mechanical stress, infection, surgical stress, and oxidant and cytokine stimulation, cells produced high levels of Heat shock protein to protect themselves against these unfavourable conditions⁴⁰ They participate in vital physiological processes in the cell such as folding, assembly, and translocation of polypeptides across membranes and play a role in protein repair after cell damage.

HSP 60 or Cpn 60 is a mitochondrial chaperonin that functions as a chaperonin to assist in folding linear amino acid chains into their respective three-dimensional structure. HSP 60 has been shown to be released from specific cells like peripheral blood mononuclear cells (PBMCs) when there are lipopolysaccharides (LPS) or GroEL present. This suggests that the cell has different receptors and responses to human and bacterial HSP 60. In addition, it has been shown that HSP 60 has the capability of activating monocytes, macrophages and dendritic cells and also of inducing secretion of a wide range of cytokines.

The HSP 60 was shown to induce a secretion of proinflammatory cytokines in professional antigen presenting cells and to enhance the activation of T cells in primary stimulation. HSP 60 is expressed on the surface of different eukaryotic cell lines increases the activation of T cells in primary stimulation. Although heat shock proteins are typically regarded as being intracellular they can be expressed on the surface of mononuclear cells⁷⁷ and HSP 60 has been identified in serum of healthy individuals⁵⁶

A significant temperature elevation up to 2°C is observed in inflamed periodontal pocket. Moreover, it is known that several pro-inflammatory immune mediators are produced in the inflamed periodontal tissue. These stressors might cause an upregulation of endogenous HSP in the periodontal tissue of periodontitis patients.

*Lundqvist C et al. (1994)*³⁸ found the expression of HSP 60 to be higher in gingival epithelial cells of inflamed tissue samples from periodontitis patients compared with samples from periodontally healthy individuals.

*Ando T et al. (1995)*² demonstrated that the serum from a periodontitis patient, contained antibodies that reacted with *A. actinomycetemcomitans*, *F. nucleatum*, and *P. nigrescens* HSP 60 and/or HSP 70, suggesting that these proteins may be involved in the pathogenesis of periodontitis.

*M.D.A. Petit et al 1999*⁵³ investigated the proliferative responses of PBMCs of patients with periodontitis (n = 10) and controls with gingivitis(n =

12) to recombinant mycobacterial HSP 60 (MycHSP60) and HSP 70 (MycHSP70), as well as recombinant human HSP 60 (HumHSP60) and HSP 70 (HumHSP70) and also the proliferative responses to *Candida albicans* and purified protein derivatives of *Mycobacterium* (PPD). Mean responses to HumHSP 60, MycHSP 60, and HumHSP 70, MycHSP 70 were significantly lower for patients compared with controls. The level of IFN- γ in the supernatants of the cells stimulated with HSP's was lower in the patients compared with controls. This concurs with the current hypothesis that periodontitis patients have a depressed Th1 response. He found that with an increasing estimated subgingival bacterial load, periodontitis patients mount a decreasing immune response to HSPs, and the poor reactivity to HSPs may be a susceptibility factor for destructive periodontal disease and may need to be considered in the pathogenesis of this condition.

*Tabeta K et al. (2000)*⁶⁹ reported that gingival tissue extracts from healthy or periodontitis patients contain antibodies to the GroEL protein of *Porphyromonas gingivalis*. They also demonstrated that diseased periodontal tissues reacted more strongly to *Porphyromonas gingivalis* GroEL and human HSP 60 compared with healthy controls. The authors showed that periodontitis patients had a higher antibody titer against *Porphyromonas gingivalis* GroEL than did healthy subjects.

*K. Ueki (2002)*⁷³ demonstrated that serum antibodies to both HumHSP 60 and *Porphyromonas gingivalis* GroEL were elevated in

periodontitis patients compared with healthy subjects. The stimulatory effect of human and bacterial HSP 60 on the production of tumour necrosis factor- α (TNF- α) was examined in phorbol myristate acetate stimulated THP-1 cells (Human monocytic cell line). The activity of HSP 60 was inhibited by anti-CD14 and anti-Toll-like receptor 4 (TLR4) antibodies, suggesting that both CD14 and TLR4 mediate HSP 60 signalling. Immunohistochemical analysis demonstrated that HSP 60 is abundantly expressed in periodontitis lesions. Therefore, it is postulated that periodontopathic bacteria stimulate the cells in the periodontium to up-regulate the expression of HSP 60, which in turn may stimulate macrophage and possibly other cells to produce proinflammatory cytokines. These mechanisms may be involved in the chronicity and tissue destruction of periodontal disease.

Hsp60 & immune Response in periodontal disease

Immune responses to bacterial HSP may generate cross reacting immunity to self-HSP and precipitate damaging inflammatory responses. Endogenous rather than bacterial HSP are more likely to be involved in signalling innate responses in periodontal disease.

Human but not periodontopathic bacterial HSP 60 can induce TNF- α production in macrophages and this activity is mediated at least in part by CD14 and TLR4, both of which are known to be LPS receptors. Bacterial homologue of HumHSP60, *Porphyromonas gingivalis* GroEL and *Aggregatibacter actinomycetemcomitans* GroEL did not show TNF- α inducing

activity. Proinflammatory cytokine production induced by autologous HSP 60 could be another pathway leading to periodontal tissue destruction.

*Petit MD et al. (1999)*⁵³ demonstrated that the level of IFN- γ production was significantly lower in the periodontitis group than in the control group. NK T cells are recruited to down-regulate the autoimmune response against self-components such as HSP 60

*Argueta JG et al (2006)*⁴ investigated whether the Toll-like receptor (TLR) family plays a functional role as a *Porphyromonas gingivalis* GroEL receptor. Human macrophage-like THP-1 cells (Human monocytic cell line) were used and the nuclear factor- κ B (NF- κ B) activity of cells stimulated with a recombinant *Porphyromonas gingivalis* GroEL was measured with a luciferase assay. Flow cytometry analysis was used to determine the binding to THP-1 cells of fluorescein isothiocyanate (FITC)-labeled GroEL. He observed by luciferase assay that the purified recombinant GroEL was able to stimulate NF- κ B transcriptional activity in THP-1 cells. Flow cytometry analysis showed that the FITC-labeled GroEL bound to THP-1 cells in a dose-dependent fashion. He concluded that *Porphyromonas gingivalis* GroEL induces its intracellular signaling cascade in Th1 cells via TLR2 or TLR4 and via a combination of both receptors.

*Fukui M et al (2006)*²⁰ stated that Salivary IgA to GroEL may have a protective role by reducing the inflammatory response induced by GroEL derived from periodontopathogenic bacteria.

*Yamazaki K et al (2002)*⁸⁰ demonstrated that HSP 60-specific T cells accumulated in the gingival lesions of periodontitis patients but not in gingivitis patients and that the T cell clones with an identical specificity to those in peripheral blood existed in periodontitis lesions.

*Choi JI et al (2004)*⁹ showed that *Porphyromonas gingivalis* HSP reactive T cell immune response might be involved in immunopathogenesis of periodontal disease. They suggested that T cells in the circulating peripheral blood may home to periodontal lesions where *Porphyromonas gingivalis* have infiltrated potentially leading to T cell response cross reactive to mammalian HSP of gingival fibroblasts.

*Wassenar A et al (1995)*⁷⁶ described the cloning and characterization of CD4 and CD8 T lymphocytes isolated from inflamed gingival tissue obtained from four patients with chronic periodontitis. Clones were raised with phytohemagglutinin and interleukin-2 and tested for proliferation in response to whole-cell antigens of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, human collagen type I, and two bacterial heat shock proteins using flow cytometry analysis. Most clones were reactive with *P. intermedia*, it seems that the immune response is not strictly directed against this particular microorganism, as clones reactive with one of the other bacteria were also obtained from two patients. He proposed that collagen-specific CD4 Th2-like T cells contribute to the chronicity of periodontitis but that their modes of activation might be

controlled by Th0-like T cells specific for periodontitis-associated bacteria, suggesting that autoimmune component might be involved in the pathogenesis of periodontitis.

*Yamazaki K, Ohsawa Y, Yoshie H. et al (2001)*⁸¹ showed that immune responses to auto antigens such as collagen Type I or HSP 60 is controlled by NK T cells through CD1 expression.

*K Yamazaki, (2004)*⁸² determined whether periodontal treatment could influence the level of serum antibodies to HumHSP 60 and Porphyromonas gingivalis GroEL. Sera were obtained from 21 patients with moderate to advanced chronic periodontitis at the baseline examination and again after completion of treatment. Antibody levels were determined using an ELISA. The mean anti-P. gingivalis GroEL antibody levels were down-regulated significantly by periodontal treatment. The mean level of anti-human HSP60 antibody remained unchanged although individual levels of antibody either increased or decreased after periodontal treatment, suggesting that synthesis of these antibodies might be regulated independently during the course of periodontal infection.

*Kazuhisa Yamazaki,(2004)*⁷⁹ he examined the proliferative response of peripheral blood mononuclear cells (PBMC), as well as the cytokine profile and T-cell clonality, for periodontitis patients and controls following stimulation with recombinant HumHSP 60 and Porphyromonas gingivalis GroEL. The nucleotide sequences within complementarity-determining region

3 of the T-cell receptor (TCR) chain were compared between HSP 60-reactive peripheral blood T cells and periodontitis lesion-infiltrating T cells. Periodontitis patients demonstrated significantly higher proliferative responses of PBMC to HumHSP 60, but not to *Porphyromonas gingivalis* GroEL, than control subjects. The response was inhibited by anti-major histocompatibility complex class II antibodies. Analysis of the nucleotide sequences of the TCR demonstrated that HumHSP 60 - reactive T-cell clones and periodontitis lesion-infiltrating T cells have the same receptors, suggesting that HSP 60-reactive T cells accumulate in periodontitis lesions. Analysis of the cytokine profile demonstrated that HSP 60-reactive PBMC produced significant levels of gamma interferon in periodontitis patients, whereas *Porphyromonas gingivalis* GroEL did not induce any skewing toward a type 1 or type 2 cytokine profile. He suggested that periodontitis patients have HumHSP 60 - reactive T cells with a type 1 cytokine profile in their peripheral blood T-cell pools.

*Ford P et al (2005)*¹⁸ examined the nature of the inflammatory infiltrate and the presence of HumHSP 60 and GroEL in 31 carotid endarterectomy specimens. HumHSP 60 expression was evident on endothelial cells and cells with the appearance of smooth muscle cells and lymphocytes

*T. Honda (2006)*²⁷ compared the gene expression profile of inflammatory mediators including proinflammatory cytokines and other

inflammatory molecules, and anti-inflammatory cytokines by using quantitative real-time polymerase chain reaction in gingivitis and periodontitis lesions. Interleukin (IL)-1 β , interferon (IFN)- γ and RANKL, Transforming growth factor (TGF)- β 1 tended to be higher in periodontitis, whereas tumour necrosis factor (TNF)- α and IL-12 , P 40, IL-10 and IL-4 showed no difference. Heat-shock protein 60 (HSP60) expression was up-regulated significantly in periodontitis. He concluded that autoimmune response to HSP 60 may exert in periodontitis lesion, and suggest that perhaps subtle differences in the balance of cytokines may result in different disease expression.

*Jarjour WN et al (1991)*²⁸, who suggested that the difference in the levels of anti-HSP antibodies seen in sera of patients with various rheumatoid and other inflammatory diseases compared to normal controls, could merely reflect disease-associated polyclonal B cell activation. Subsequent investigations have suggested that such antibodies or specific T cells against HSP were associated with immune and autoimmune diseases.

*K Tabeta (2000)*⁶⁸ examined the presence of antibodies to the 60-kD human and *Porphyromonas gingivalis* GroEL HSP 60 in the sera and inflamed gingival tissues of periodontitis patients. Western blot analysis clearly demonstrated that the number of periodontitis patients showing a positive response to *Porphyromonas gingivalis* GroEL and it was higher than the number of periodontally healthy subjects. For HumHSP 60, a higher frequency

of seropositivity was found in the periodontitis patients than in the healthy subjects. In addition, the periodontitis patients demonstrated stronger reactivity compared with the healthy subjects. Quantitative analysis of serum antibodies by ELISA also demonstrated that the levels of antibodies in the sera of patients were significantly higher than those of control subjects. He suggested that molecular mimicry between GroEL of the periodontopathic bacterium *Porphyromonas gingivalis* and autologous HumHSP 60 may play some role in immune mechanisms in periodontitis.

*Young RA and Elliott TJ (1989)*⁸³ showed that through these cross-reactive epitopes, T-cells with specificity for self-HSP can be activated during infection. Indeed, conserved HSP epitopes may trigger self-HSP reactive T-cells, with disease-suppressive regulatory potential.

Methods of Hsp60 Detection in Periodontitis

The increasing interest in HSP as markers of exposure to environmental stress or diseases requires a generally applicable method for HSP determination. There are many Classical methods that evaluate HSP at the protein level

Enzyme-linked immunosorbent assay

It is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. In ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is

washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA).

After the antigen is immobilized the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bioconjugation.

Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. Older ELISAs utilize chromogenic substrates, though newer assays employ fluorogenic substrates enabling much higher sensitivity. ELISA is used to detect serum antibodies to periodontal pathogens and has been used in recent studies to quantify specific pathogens.

*Handley HH et al (1995)*²⁴ has examined by ELISA the serum IgG antibody levels to McyHSP 65 and HumHSP 60, as well as to the *Escherichia coli* HSP 60, GroEL, in patients with Rheumatoid arthritis, Systemic lupus erythematosus (SLE), Reiter's syndrome, active tuberculosis, and normal controls.

*Tabeta K et al (2001)*⁶⁹ demonstrated the presence of antibodies to the HSP 60 of *Actinobacillus actinomycetemcomitans* in the sera of periodontitis patients and periodontally healthy control subjects by enzyme-linked immunosorbent assay using a recombinant *A. actinomycetemcomitans* GroEL as an antigen.

*Watanabe S et al (2003)*⁷⁶ established an ELISA system to measure anti-HSP 60 IgG and IgA antibody titer in sera of Rheumatoid arthritis patients

*Goretzk Li et al (2006)*²² stated that immunoassays for the quantification of the HSP biological markers help to define more precisely the significance of heat shock proteins in cancer.

*Shamaei-Tousi A et al (2007)*⁶³ measured plasma levels of HSP10, HSP60 by immunoassay and related to other plasma measures of inflammation.

Western Blot / Immunoblotting

Many authors have demonstrated the expression of human and bacterial HSP 60 by western blot ^{64,55,36,37,2,74} It is a powerful method for detecting a particular protein in a complex mixture which combines the superior resolving power of gel electrophoresis, the specificity of antibodies, and the sensitivity of enzyme assays. This multistep procedure is commonly used to separate proteins and then identify a specific protein of interest. Two different antibodies are used in this method, one specific for the desired protein and the other linked to a reporter enzyme. It is a technique for the analysis of proteins within the cell. Proteins are electrophoresed through a SDS polyacrylamide gel so as to separate the molecules according to size, and are then transferred to a membrane and hybridized with an antibody against the specific protein of interest.

Systemic inflammatory response in periodontal disease:-

Chronic periodontal infections are associated with systemic changes to blood and blood forming organs. Periodontal disease is capable of predisposing to vascular disease given the abundance of gram negative species involved, easily detectable levels of proinflammatory cytokines, dense immune cell infiltrate involved, association of peripheral fibrinogen and white cell count, extent and chronicity of disease.

Lipopolysaccharide is released as extra cellular blebs from micro organisms within the periodontal pocket and may enter diseased periodontium. Bacteraemia may result in free LPS from periopathogenic bacteria being present in plasma. Bacteraemia results in gram negative micro organisms entering the circulation, where they might activate leucocytes, platelets or endothelium directly.

It has been hypothesised that certain individuals may respond to a microbial challenge with an over exuberant or hyper reactive inflammatory response. This may be demonstrated by an increase in release of proinflammatory mediators when challenged by bacterial LPS. Peripheral blood monocytes from such individuals secrete 3-10 times more inflammatory mediators in response to bacterial LPS than normal individuals.

According to **Kinane DF and Lowe GDO (2000)**³² Hyper reactive mononuclear phagocyte expression may be constitutive in susceptible individuals or may be induced in those with infections such as periodontal disease. Patients with periodontitis have peripheral monocytes that, when reacting with LPS give increased prostaglandin production. Hyper responsiveness may be induced by persistent infection of the periodontium, and this may have a role in the mechanistic link between periodontitis and atheroma formation.

Periodontitis can elicit a systemic inflammatory response by activating the hepatic acute phase response. This occurs presumable as a consequence of systemic appearance of transient and recurrent bacteremia of oral origin, which has been a long recognised characteristic of periodontal infections.

Offenbacher S et al (1998)⁴⁶ hypothesised that gram negative anaerobe pathogens from periodontium trigger release of biologically active mediators such as PGE₂, and TNF α in circulation causing premature labour.

According to **Williams RC, Offenbacher SI (2000)⁴⁷** Cross sectional evidence indicates that periodontitis elicits a mild elevation in markers of acute phase response including C reactive protein, haptoglobin, alpha 1 antitrypsin, and fibrinogen. The liver in response to systemic challenge of organisms secretes acute phase proteins. This acute phase response is triggered by blood borne oral LPS and oral bacteria which elicit release of cytokines IL-6, TNF α . Markers of acute phase response associated with periodontitis and cardiovascular risk include C reactive protein, increase in WBC count, and increase in α 1 anti trypsin, haptoglobin and fibrinogen and decrease in albumin

Noack B et al (2001)⁴⁵ determined that CRP plasma levels are increased in periodontitis. Also, there are elevated levels of CRP associated with infection with subgingival organisms often associated with periodontal disease, including Porphyromonas gingivalis, Prevotella Intermedia, Campylobacter rectus, Bacteriodes forsythus.

Havemose-Poulsen A et al (2005)²⁵ reported an increase in the proinflammatory cytokines including IL-1 α , IL-1 β , IL-RA, IL-6, IL-10, TNF α levels in systemic circulation following periodontal disease

Periodontal disease and circulating HSP 60 levels:-

T-cell immune responses specific to bacterial or HumHSP 60 have been demonstrated in atherosclerosis. The host immune system primed by HSP of a major periodontal pathogen, such as *Porphyromonas gingivalis*, can cross-react with its cognate mammalian counterpart in gingival connective tissue or arterial walls.

Wand-Wurtenberger A, (1991)⁷⁷ demonstrated that although HSP are typically regarded as being intracellular, they can be expressed on the surface of mononuclear cells and stressed aortic endothelial cells.

Roman Kleindienst, in (1993)³³ demonstrated that the intensity of HSP 60 expression correlates positively with the atherosclerotic severity and that most lymphocytes participating in atherogenesis bear the α or β TCR, although γ or δ TCR+ cells are also enriched in atherosclerotic lesions. Expression of HSP 60 by intimal cells, caused, eg, by hemodynamic shear forces, may be responsible for recruitment of HSP-sensitized T cells, thus leading to the induction of an initiating inflammatory process in atherosclerosis. Other risk factors, such as high serum cholesterol levels, contribute to the final outcome of the disease.

A. Graham Pockley (2000)⁵⁷ demonstrated HSP 60 was present in all of the patients with Boderline Hypertension, Circulating HSP 70 was detectable in 36 of the 65 individuals with Boderline Hypertension and 37 of the 75 control subjects. Anti– HumHSP 60, anti– HumHSP 60, and anti– MycHSP 65 antibodies were detected in all of the Boderline Hypertension samples analyzed and also he analysed the relationships Among HSP, Anti-HSP antibodies,I/M Thickness, Blood Pressure Levels, and Metabolic Variables. There were significant associations between HSP 60 and Diastolic Blood Pressure and 24-hour systolic BP and there was no significant association between HSP or HSP antibody levels and BP parameters when the Boderline Hypertension and Normotensive groups were analyzed separately.

Amir Kol (2000)³⁴ He hypothesised that HSP 60 activates mononuclear cells and macrophages through CD14 signaling. As a measure of cellular activation, he assessed the cellular production of IL-6, an important proinflammatory cytokine and mediator of the acute phase response HumHSP 60 induced the synthesis of IL-6 by PBMC. HSP 60 induced IL-6 similarly to optimal concentrations of Escherichia coli LPS. Preincubation of PBMC with an anti-CD14 Ab blocked IL-6 production in response to either HSP 60 or Escherichia Coli LPS. The anti-CD14 Antibody did not inhibit this response thus he demonstrated that CD14 mediates cellular activation induced by HumHSP 60.

*Qingbo Xu et al (2000)*⁷⁸ demonstrated that markers of inflammation (C-reactive protein or soluble ICAM-1) and the presence of chronic infections are strongly associated with the development of atherosclerosis in the carotid arteries and also he demonstrated that the predictive significance of serum HSP 60 for atherosclerosis progression grows with laboratory and/or clinical evidence of chronic infection/inflammation.

*Pockley, A. Graham et al (2000)*⁵⁷ demonstrated that HSP 60, HSP 70 and anti-HSP 60 antibody levels in hypertension were similar to those in normotensive controls, whereas anti-HSP 70 and anti-HSP 65 antibody levels were elevated HSP 60 levels and atherosclerosis were not associated. Anti-HSP 70 and anti-HSP 65 antibody levels were both associated with hypertension, independently of age, smoking habits and blood lipids.

*Hannes Perschinka(2003)*⁵² purified serum antibodies to Escherichia coli HSP 60 (GroEL), the 60-kD Chlamydial HSP, and HSP 65 of Mycobacterium tuberculosis by affinity chromatography from clinically healthy subjects with sonographically proven carotid atherosclerosis. Reactivity of the purified antibodies with overlapping HumHSP 60 peptides was measured, and 8 shared common epitopes, recognized by all anti-bacterial HSP 60/65 antibodies, were identified. Antisera specific for these cross-reactive epitopes were produced by immunizing rabbits with peptides derived from HumHSP 60. By immunohistochemistry, the epitopes were found to be present in the arterial wall of young subjects during the earliest stages of the

disease. Antibodies to microbial HSP 60/65 recognize specific epitopes on HumHSP 60. These cross-reactive epitopes were shown to serve as autoimmune targets in incipient atherosclerosis and might provide further insights into the mechanisms of early atherogenesis.

Chung SW et al (2003)¹⁰ evaluated the recognition of *Porphyromonas gingivalis* HSP 60 and HumHSP 60 by immune sera in *P. gingivalis*-infected periodontitis and atherosclerosis patients. Mononuclear cells from atheroma lesions were stimulated with *Porphyromonas gingivalis* HSP and sera from periodontitis or atherosclerosis patients were subjected to Western immunoblotting to *Porphyromonas gingivalis* HSP or HumHSP, respectively. Western immunoblot analysis demonstrated the dual reactivity of anti-*Porphyromonas gingivalis* antisera with *Porphyromonas gingivalis* HSP and HumHSP. We could also establish *Porphyromonas gingivalis* HSP - specific T cell lines from the atheroma lesions, a mixture of CD4⁺ and CD8⁺ cells producing the cytokines characteristic of both Th1 and Th2 subsets. He suggested the modulating effect of *Porphyromonas gingivalis* HSP 60 in the immunopathogenesis of periodontitis and atherosclerosis.

J. I. Choi in (2004)⁹ Identified the T- and/or cross-reactive B-cell epitopes of *Porphyromonas gingivalis* and HumHSP 60 in atherosclerosis patients. Periodontitis patients with elevated IgG antibody responses to only *Porphyromonas gingivalis* were included in the study. T-cell epitopes of *Porphyromonas gingivalis* HSP 60 were identified with the use of previously

established *Porphyromonas gingivalis* HSP-reactive T-cell lines. B-cell epitopes of *P. Gingivalis* HSP 60 and HumHSP 60 were identified by the use of patients' sera. Anti-*Porphyromonas gingivalis* , anti-*Porphyromonas gingivalis* HSP 60, or anti- HumHSP 60 ,IgG antibody titers were higher in the atherosclerosis patients compared with the healthy subjects. He concluded that *Porphyromonas gingivalis* HSP 60 might be involved in the immunoregulatory process of atherosclerosis, with common T- and/or B-cell epitope specificities and cross-reactivity with HumHSP 60

J. I. Choi (2004)⁸ evaluated the T-cell immune responses specific to *Porphyromonas gingivalis* HSP 60 in patients suffering from atherosclerosis. Anti- *Porphyromonas gingivalis* HSP 60 IgG antibody titers were elevated in all patients. *Porphyromonas gingivalis* HSP -specific T-cell lines from the atheroma lesions and the peripheral blood has been established. The T-cell lines were a mixture of CD4⁺ and CD8⁺ cells producing the cytokines characteristic of both Th1 and Th2 subsets. He suggested that the T-cell immune response specific to *Porphyromonas gingivalis* HSP 60 may be involved in the immunopathologic process of atherosclerotic diseases.

Yamazaki K (2004)⁷⁹ Compared the cellular and humoral immune responses to HSP 60 in atherosclerosis patients and periodontitis patients and healthy subjects using human and *Porphyromonas gingivalis* HSP 60 (GroEL) as antigens. Antibody levels to both human and *Porphyromonas gingivalis* HSP 60s were the highest in atherosclerosis patients, followed by periodontitis

patients and healthy subjects. Clonal analysis of the T cells clearly demonstrated the presence of not only HumHSP 60 - but also Porphyromonas gingivalis GroEL-reactive T-cell populations in the peripheral circulation of atherosclerosis patients. Furthermore, these HSP 60-reactive T cells seemed to be present in atherosclerotic lesions in some patients. These results suggest that T-cell clones with the same specificity may be involved in the pathogenesis of the different diseases.

*Qingzhong Xiao, MD (2005)*⁵⁸ tested whether soluble HSP60 levels are associated with the progression of carotid arteriosclerosis, prospectively. The association of soluble HSP60 with early atherogenesis (5-year development and progression of nonstenotic carotid plaques) was investigated. The follow-up period was between 1995 and 2000 and, included 684 subjects. Soluble HSP60 levels measured in 1995 and 2000 were highly correlated indicating consistency over a 5-year period. Circulating HSP60 levels were significantly correlated with anti-lipopolysaccharide and anti-HSP60 antibodies. HSP60 levels were significantly associated with early atherogenesis, both in the entire population. The risk of early atherogenesis was additionally amplified when high- soluble HSP60 and chronic infection were present together. He confirmed the association between high levels of soluble HSP60 and early carotid atherosclerosis. This possibly indicates an involvement of soluble HSP60 in activating proinflammatory processes associated with early vessel pathology.

Ford P et al (2006)¹⁸ demonstrated presence of bacteria, including periodontopathogens in atherosclerotic plaques and suggested that cross-reactivity of the immune response to bacterial GroEL with HumHSP 60 is a link between infections and atherosclerosis.

Alireza Shamaei-Tousi et al (2007)⁶³ measured the plasma levels of HSP10, HSP60 by immunoassay and related to other plasma measures of inflammation in periodontal patients and matched controls and also at 1 day and 6 months following periodontal or control therapy. Periodontal patients had significantly less circulating levels of HSP10 compared with the controls. In contrast, more periodontal patients had intermediate levels of HSP60. Treatment of the periodontitis caused an increase in plasma levels of HSP10. Treatment had no influence of HSP60 levels. Plasma HSP10 levels after therapy correlated with markers of periodontal clinical improvement. He concluded that circulating levels of molecular chaperones are influenced by local inflammation. The marked decrease of this circulating protein in active inflammation and its recovery post-treatment suggested that it may have a role in controlling periodontal inflammation.

Salivary markers of periodontal inflammation:-

Saliva is a fluid that is readily available and contains locally-produced microbial and host response mediators, as well as systemic (serum) markers that may prove to be an aid in the diagnosis of periodontal disease and systemic disease. Salivary markers that have been studied as potential

diagnostic tests for periodontal disease include proteins of host origin (i.e., enzymes, immunoglobulins), phenotypic markers, host cells, hormones (cortisol), bacteria and bacterial products, ions and volatile compounds. Saliva has been proposed as a noninvasive diagnostic fluid that could not only be used to help diagnose oral diseases, but as the body's mirror that would also have application in the diagnosis of systemic conditions.

The levels of salivary biomarkers, such as cytokines, could potentially be used as a surrogate to distinguish periodontally healthy individuals from subjects with periodontitis. Furthermore analysis of saliva may offer a cost-effective approach to assessment of periodontal disease in large populations. In spite of the recognized diagnostic potential of saliva, only a few reports have attempted to correlate the levels of cytokines in saliva with the periodontal condition of the subjects.

*Miller et al (2006)*³⁹ conducted a study to determine if salivary biomarkers specific for three aspects of periodontitis, namely inflammation, collagen degradation and bone turnover, correlated with clinical features of periodontal disease. He reported that the mean levels of IL 1 and MMP 8 in saliva were significantly higher in subjects with periodontitis than in periodontally healthy controls.

*Ruhl (2004)*⁶² measured the levels of IL 1, IL 6, IL 8, epidermal growth factor, nerve growth factor. He found that IL 1, IL 6, IL 8 were present

in whole saliva at concentrations significantly higher than in major salivary gland secretions.

Teles RP (2009)⁷⁰ determined whether the levels of 10 different cytokines in saliva differed between a group of periodontally healthy individuals and a group of subjects with periodontitis. Correlations between the concentrations of these 10 cytokines and clinical parameters of periodontal disease were also examined. These cytokines are measured in whole saliva using a multiplexed bead immunoassay (Luminex). Mean salivary levels of granulocyte-macrophage colony-stimulating factor, interleukin-1beta, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interferon-gamma and tumor necrosis factor-alpha could not discriminate between periodontal health and disease.

Andrej Aurer (2005)⁶ measured the concentration of host inflammatory proteins: C-reactive protein (CRP), C3 and C4 complement components, alpha-2-macroglobulin and tumor-necrosis factor in unstimulated saliva of periodontally health, edentulous persons, patients with chronic periodontitis and in patients with aggressive periodontitis. TNF was below the level of detection in all samples except one. Edentulous persons and patients with chronic periodontitis had significantly reduced concentrations of CRP, C3 and alpha-2-macroglobulin. Edentulous persons and aggressive periodontitis patients had lower C4 concentrations. He concluded that edentulous persons and chronic periodontitis patients have reduced salivary

concentrations of host inflammatory proteins and also suggested that a reduction in host responsiveness might play a role in the pathogenesis of chronic periodontitis.

Tatjana Todorovic et al (2006)⁷² examined the activity of aspartate and alanine aminotransferase (AST, ALT), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline and acidic phosphatase (ALP, ACP), gamma glutamyl transferase (GGT) in saliva from patients with periodontal disease before and after periodontal treatment and in saliva from healthy patients. Patients with periodontal disease were under conventional periodontal treatment. There was statistically significant increase of activity of aspartate and alanine aminotransferase (AST, ALT), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline and acidic phosphatase (ALP, ACP), gamma glutamyl transferase (GGT) in saliva from patients with periodontal disease in relation to control group and also a positive correlation between the activity of examined salivary enzymes and value of the gingival index. After conventional periodontal therapy the activity of all salivary enzymes was significantly decreased. He concluded that activity of these enzymes in saliva, as biochemical markers for periodontal tissue damage, may be useful in diagnosis, prognosis and evaluation of therapy effects in periodontal disease.

Balwant Rai et al (2008)⁵⁹ estimated salivary TNF α levels in periodontitis and healthy normal. Significantly higher levels Salivary TNF α was observed in periodontitis patients as compared to controls ($p < 0.001$). He

concluded that Saliva provides an ideal medium for the detection of proinflammatory markers of the oral cavity. Salivary TNF α analysis may be a useful diagnostic tool and a potential prognostic marker in periodontal disease.

Patricia Yen Bee Ng (2007)⁵⁰ evaluated the association between radiographic evidence of alveolar bone loss and the concentration of host-derived bone resorptive factors (interleukin-1 beta, TNF α , interleukin-6, prostaglandin-E2), and markers of bone turnover [pyridinoline crosslinked carboxyterminal telopeptide of type I collagen (ICTP), osteocalcin, osteonectin] in stimulated human whole saliva collected from untreated dental patients. Alveolar bone loss scores for each patient were derived from radiographic examination. Variables positively associated with increased bone loss score were: age, current smoking, use of bisphosphonate drugs, and salivary interleukin-1beta levels above the median. Salivary osteonectin levels above the median were associated with a decreased bone loss score. Additional in vitro studies were carried out to determine the fate of interleukin-1beta, interleukin-6 and TNF- α added to whole and parotid saliva. All cytokines added to saliva were detected in significantly lower concentrations than when added to buffer alone. He concluded that detection of biomarkers by conventional immunoassays may underestimate the actual quantity of molecules in saliva.

Diagnostic methods used in clinical practice today lack the ability to detect the onset of inflammation and to identify those patients who are

susceptibility to future disease progression. oral fluid based point of care(POC) diagnostics are commonly used in medicine and, more recently, are being adapted for the potential “ chair side “ determination of oral diseases.the latest clinical applications use new” lab-on-a-chip”(LOC) technologies as rapid POC diagnostic test for systemic infectious diseases and periodontal disease.²⁶ The development of rapid Point of Care chair side diagnostics has the potential for the early detection of periodontal infection and progression to identify the incipient disease.

*Amy e. Herr, Anson v. hatch, William v. Giannobile (2007)*²⁶ developed a portable microfluidic device for detection of potential biomarkers of periodontal disease in saliva. The device performs rapid microfluidic chip-based immunoassays (<3–10 min) with low sample volume requirements (10 µL) and appreciable sensitivity. This method facilitates hands-free saliva analysis by integrating sample pretreatment (filtering, enrichment, mixing) with electrophoretic immunoassays to quickly measure analyte concentrations in minimally pre treated saliva samples. The microfluidic chip has been integrated with miniaturized electronics, optical elements, such as diode lasers, fluid-handling components, and data acquisition software to develop a portable, self-contained device. The device and methods are being tested by detecting potential biomarkers in saliva samples from patients diagnosed with periodontal disease. The microchip-based analysis can readily be extended to detection of biomarkers of other diseases, both oral and systemic, in saliva and

Heat shock proteins in saliva:-

Molecular chaperones were considered to be intracellular, but there is increasing evidence demonstrating their cytoprotective and immune modulator properties outside the cell, exerting cytokine-like effects and influencing immune recognition. There is scant evidence regarding HSP 60 in saliva. However the major extracellular chaperone HSP 70 was found in saliva, indicating a possible effect of HSP 70 on mucosal surfaces. HSP 70 has been found to be present in human blood sera. Cpn10 and Cpn60 are present in pancreatic juice, but HSP 70 is not. These observations raise the possibility that molecular chaperones may be present in other secretory fluids, such as human saliva.

Fabian TK et al (2007)¹⁵ summarized the immune-modulatory role of the 70-kDa stress protein family, with special attention on the potential impact of salivary HSP 70 on oral defense mechanisms. Three major facets of HSP 70-induced immune activation are : 1) the appearance of HSP 70 on the surface of certain tumor cells or virally infected cells, leading to their phagocytosis and subsequent lysis; 2) the role of extracellular uncomplexed HSP 70 as a danger signal, leading to the secretion of proinflammatory cytokines from antigen-presenting cells and T lymphocytes and of nitric oxide from macrophages as well as to complement activation; 3) receptor-mediated uptake of peptide-loaded HSP 70 to antigen-presenting cells and cross-presentation of the HSP 70-peptide complex as an antigen to cytotoxic T cells

and natural killer lymphocytes. The immune-activating effect of salivary HSP 70 may also be highly important in oral defense, especially in areas where molecular and cellular participants of the immune response appear on the surface of the oral cavity (i.e. several lesions of the mucosa and the periodontal tissues).

Fabian TK (2003)¹⁶ Human whole saliva was collected from six participants under resting conditions and secretory stimulation. The samples were precleared by centrifugation and sterile filtered. Salivary volume, protein concentration and amylase activity were determined. For detection of HSP 70 saliva proteins were separated on a 12.5% SDS polyacrylamide gel. Semi-dry Western blot analysis was used with a primary antibody against the inducible form of HSP 70. There was a significant decrease of HSP 70, and a non-significant decrease of total protein concentration during stimulation, whereas the activity of salivary amylase increased significantly. Stimulation significantly increased the HSP 70, total protein and amylase outputs as well as the amylase/protein ratio, and decreased the HSP 70/amylase and HSP 70/protein ratios. He concluded that HSP 70 is secreted to saliva, but unlike amylase is not transported by the exocytotic secretory mechanisms of acinar cells. Passive transport mechanisms of HSP 70 from blood serum or from salivary gland cells may be major routes of salivary HSP 70 secretion.

Fejerdy L (2004)¹⁷ investigated whether repeated, short-term heat and mechanical stimulation of the salivary glands can specifically modify the

salivary HSP 70 concentration in the human whole saliva. Both kind of stimulation increased the secretory rate significantly, during stimulation, but it decreased to control level in resting phases. HSP 70 concentration increased after the first stimulation in the case of mechanical stress and after the second stimulation in the case of heat stimulation. In contrast, a significant confluent increase of total protein concentration and amylase activity occurred after the first stimulation in the case of heat stimulation and after the second stimulation in the case of mechanical stress.

MATERIALS AND METHODS

Study Population

45 patients (21 males and 24 females) who attended the out patient Department of periodontology, Ragas Dental College and Hospitals, Chennai were enrolled in the study. Patients were divided into two groups based on their periodontal health status. Informed consent was obtained from all the patients. The patients were informed that this research work was in no way directly related to the therapy or cure of the disease. The study was undertaken following approval from the institutional review board.

Selection criteria

Group A: Healthy Gingiva - Patients exhibiting no signs of periodontal disease, determined by the absence of clinical attachment loss, absence of bleeding on probing. PPD <3mm.

Group B: Periodontitis - Patients with teeth exhibiting PPD ≥ 5 mm and CAL ≥ 3 mm and radiographic evidence of bone loss in atleast 6 teeth.⁵

Exclusion criteria

- Patients with history of periodontal therapy or antibiotic therapy in the past 6 months

- Patients with history of systemic diseases that may affect the periodontal status
- Pregnancy and Lactation
- Smokers
- Evidence of any other active oral infections eg: pulpal pathology.

Clinical evaluation:-

Clinical evaluation was done using mouth mirror and William's periodontal probe. The probing depth, clinical attachment loss, bleeding on probing was evaluated. From the above measurements, biofilm gingival interface index (BGI) was calculated as follows,

BGI-Healthy (BGI-H) - probing depth \leq 3mm and $<$ 10 %
bleeding on probing

BGI- Gingivitis (BGI-G) - probing depth \leq 3mm and \geq 10 %
bleeding on probing

BGI- Deep lesion / low bleeding - one or more sites with probing
(BGI-DL/LB) depth \geq 4mm and $<$ 10 % bleeding on
probing

BGI- Deep lesion / moderate bleeding - one or more sites with probing depth
(BGI-DL/MB) \geq 4mm and 10 to 50 % bleeding on
probing

BGI- Deep lesion / severe bleeding - one or more sites with probing
(BGI-DL/SB) depth \geq 4mm and \geq 50 % bleeding on
probing

Sample collection

3 ml of Peripheral blood drawn from patients using venepuncture from the antecubital fossa to be used for ELISA analysis as well as for total and differential white blood cell count estimations.

Peripheral blood was drawn prior to onset of Phase I periodontal therapy in periodontitis patients. All patients underwent a complete physical examination and hematological investigation in Ragas General Hospital to rule out systemic diseases.

Saliva collection:-

Salivary collection is done according to the technique by *Navazesh et al (2008)*⁴³. The patients were advised to refrain from intake of any food or beverage (water exempted) one hour before the test session. The subjects were advised to rinse his or her mouth several times with distilled water and then to relax for five minutes. The patient is asked to lean the head forward over the container with the mouth slightly open and allow the saliva to drain into the container with the eyes open. The time lasted for saliva collection is five minutes, the saliva is collected in a sterile disposable plastic container and the samples were stored at -70° C and used for further analysis.

Armamentarium

1. Disposable needle and syringe
2. Vacutainers
3. Test Tubes
4. Centrifuge Tubes
5. Micropipettes
6. Micropipette tips
7. Pasteur pipette
8. Laboratory Centrifuge
9. Refrigerator
10. Autoclavable Scott Duran bottles
11. Autoclavable containers for saliva collection
12. Haemocytometer
13. Electronic balance
14. Light Microscope
15. Ice pack (for transfer)

Reagents Required:-

1. Phosphate Buffered Saline (freshly prepared)

Formulation:

- Sodium chloride (NaCl) : 8g

- Potassium Dihydrogen Phosphate (KH_2PO_4) : 200mg
- Disodium Hydrogen Phosphate (Na_2HPO_4) : 2.9g
- Potassium Chloride(KCl) : 500 mg
- Distilled Water(H_2O) : 1 litre

2. Histopaque- 1077 – For Isolation of Mononuclear Cells

Solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/ml.

3. Distilled water

4. HSP 60 ELISA KIT(ASSAY DESIGNS)

Serum sample preparation:-

3ml of blood was drawn by vene puncture in anticubital fossa. 2ml of blood was allowed to clot at room temperature for 30 mins & centrifuged at 3000 rpm for 10 mins. Serum was then divided into 2 aliquots and transferred to labelled poly propylene tubes and stored at -70°C and used for further analysis.

Cell lysate preparation:-

Out of 3 ml; 1 ml of the Sample was collected into vaccutainers containing anticoagulant (EDTA). 1ml of blood was added to 2ml of phosphate

buffer saline(PBS).this mixture was carefully layered in a centrifuge tube containing 3ml of Histopaque -1077 (Sigma Aldrich) centrifugation at 3000 rpm for 30 mins. The buffy coat layer containing lymphocytes and mononuclear cells are separated and washed twice with 10ml PBS at 2000 rpm for 10 mins. 1ml 1x extraction reagent was added per 1ml of blood. The extract was incubated for 30 mins on ice. The extract was centrifuged in polypropylene microcentrifuge tube at 21000 rpm for mins in a refrigerated microfuge (Remi) at 4⁰ c. The supernatants were obtained and stored at -70⁰c and used for further analysis.

Enzyme linked immunoassay for HSP 60:-

Serum, cell lysate and saliva samples were diluted in sample Diluent(1:50)

- Six polypropylene tube each with one of the following standard values was prepared.100mg/ml, 50mg/ml., 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml.
- To the 96 pre coated micro litre wells ,100 micro ml of prepared HSP 60 standard (Tube #1 to Tube#2) ,sample and zero standard (0mg/ml) was added and incubated at room temperature for 60 mins.
- Liquid from all the wells were aspirated and washed with 300microliter of 1x wash buffer for 6 times using automatic washer.
- After the 6th wash, 100 micro litre of previously diluted Anti HSP 60(Goat polyclonal antibody) was added to each well and incubated at

room temperature for 60 mins .the plates were washed with wash buffer as described previously.

- 100 micro litre of previously diluted HRP (Horse radish peroxidise) conjugate was added to each well excepting the blank, and incubated at room temperature for 30 mins .plates were washed with wash buffer for 6 times.
- 100 micro litre of TMB(Tetramethylbenzidine) substrate was added to every well colour developed was visible within 1minute of addition to the wells.
- Finally 100 micro litre of stop solution 2 was added to the wells.
- Microplate reader was set according to the manufacturers instruction at a wavelength of 450 nm and absorbance was measured.

Measurement of High Sensitivity C- Reactive Protein (hs-CRP):-

Measurement of hs-CRP was performed using a immunoturbidimetric assay performed on a Randox Daytoner analyzer (Randox Laboratories, Crumlin Co., Autrium, UK). The assay range was 0.1 to 20 mg/l and detection limit was 0.03 mg/l.

Statistical analysis:-

The circulatory HSP 60 levels were obtained by adding cell lysate HSP levels and serum HSP 60 levels. The circulatory and salivary levels of HSP 60 in periodontal health and disease was compared by calculating the mean and standard deviation for each group. Student T test was used for statistical analysis and P value was calculated. Correlation coefficient of serum, saliva and cell lysates of HSP 60 in periodontal health and disease was analysed using Pearson's correlation. $P < 0.05$ was considered to be statistically significant at the 5% level.

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3 ml of Peripheral blood drawn from patients using venepuncture from the antecubital fossa to be used for ELISA analysis as well as for total and differential white blood cell count estimations.

Peripheral blood was drawn prior to onset of Phase I periodontal therapy in periodontitis patients. All patients underwent a complete physical examination and hematological investigation in Ragas General Hospital to rule out systemic diseases.

Saliva collection:-

Salivary collection is done according to the technique by *Navazesh et al (2008)*⁴³. The patients were advised to refrain from intake of any food or beverage (water exempted) one hour before the test session. The subjects were advised to rinse his or her mouth several times with distilled water and then to relax for five minutes. The patient is asked to lean the head forward over the container with the mouth slightly open and allow the saliva to drain into the container with the eyes open. The time lasted for saliva collection is five minutes, the saliva is collected in a sterile disposable plastic container and the samples were stored at -70° C and used for further analysis.

Armamentarium

1. Disposable needle and syringe
2. Vacutainers
3. Test Tubes
4. Centrifuge Tubes
5. Micropipettes
6. Micropipette tips
7. Pasteur pipette
8. Laboratory Centrifuge
9. Refrigerator
10. Autoclavable Scott Duran bottles
11. Autoclavable containers for saliva collection
12. Haemocytometer
13. Electronic balance
14. Light Microscope
15. Ice pack (for transfer)

Reagents Required:-

1. Phosphate Buffered Saline (freshly prepared)

Formulation:

- Sodium chloride (NaCl) : 8g

- Potassium Dihydrogen Phosphate (KH_2PO_4) : 200mg
- Disodium Hydrogen Phosphate (Na_2HPO_4) :2.9g
- Potassium Chloride(KCl) : 500 mg
- Distilled Water(H_2O) : 1 litre

2. Histopaque- 1077 – For Isolation of Mononuclear Cells

Solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/ml.

3. Distilled water

4. HSP 60 ELISA KIT(ASSAY DESIGNS)

Serum sample preparation:-

3ml of blood was drawn by vene puncture in anticubital fossa. 2ml of blood was allowed to clot at room temperature for 30 mins & centrifuged at 3000 rpm for 10 mins. Serum was then divided into 2 aliquots and transferred to labelled poly propylene tubes and stored at -70°C and used for further analysis.

Cell lysate preparation:-

Out of 3 ml; 1 ml of the Sample was collected into vaccutainers containing anticoagulant (EDTA). 1ml of blood was added to 2ml of phosphate

buffer saline(PBS).this mixture was carefully layered in a centrifuge tube containing 3ml of Histopaque -1077 (Sigma Aldrich) centrifugation at 3000 rpm for 30 mins. The buffy coat layer containing lymphocytes and mononuclear cells are separated and washed twice with 10ml PBS at 2000 rpm for 10 mins. 1ml 1x extraction reagent was added per 1ml of blood. The extract was incubated for 30 mins on ice. The extract was centrifuged in polypropylene microcentrifuge tube at 21000 rpm for mins in a refrigerated microfuge (Remi) at 4⁰ c. The supernatants were obtained and stored at -70⁰c and used for further analysis.

Enzyme linked immunoassay for HSP 60:-

Serum, cell lysate and saliva samples were diluted in sample Diluent(1:50)

- Six polypropylene tube each with one of the following standard values was prepared.100mg/ml, 50mg/ml., 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml.
- To the 96 pre coated micro litre wells ,100 micro ml of prepared HSP 60 standard (Tube #1 to Tube#2) ,sample and zero standard (0mg/ml) was added and incubated at room temperature for 60 mins.
- Liquid from all the wells were aspirated and washed with 300microliter of 1x wash buffer for 6 times using automatic washer.
- After the 6th wash, 100 micro litre of previously diluted Anti HSP 60(Goat polyclonal antibody) was added to each well and incubated at

room temperature for 60 mins .the plates were washed with wash buffer as described previously.

- 100 micro litre of previously diluted HRP (Horse radish peroxidise) conjugate was added to each well excepting the blank, and incubated at room temperature for 30 mins .plates were washed with wash buffer for 6 times.
- 100 micro litre of TMB(Tetramethylbenzidine) substrate was added to every well colour developed was visible within 1minute of addition to the wells.
- Finally 100 micro litre of stop solution 2 was added to the wells.
- Microplate reader was set according to the manufacturers instruction at a wavelength of 450 nm and absorbance was measured.

Measurement of High Sensitivity C- Reactive Protein (hs-CRP):-

Measurement of hs-CRP was performed using a immunoturbidimetric assay performed on a Randox Daytoner analyzer (Randox Laboratories, Crumlin Co., Atrium, UK). The assay range was 0.1 to 20 mg/l and detection limit was 0.03 mg/l.

Statistical analysis:-

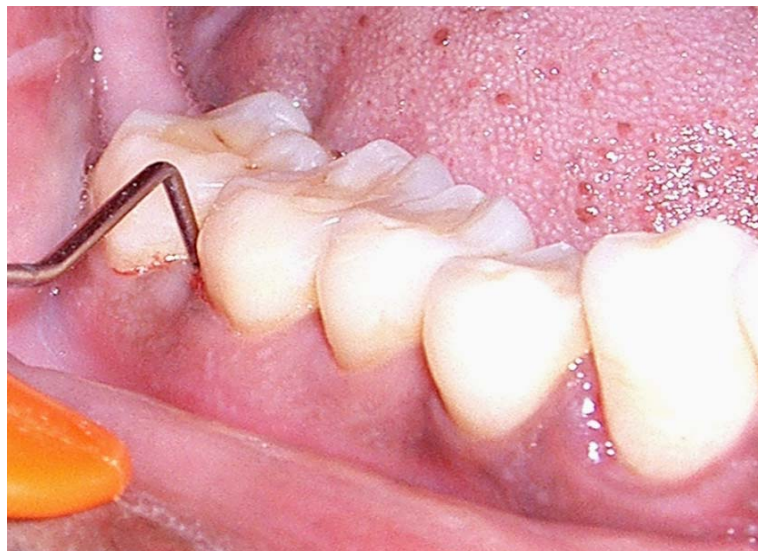
The circulatory HSP 60 levels were obtained by adding cell lysate HSP levels and serum HSP 60 levels. The circulatory and salivary levels of HSP 60 in periodontal health and disease was compared by calculating the mean and standard deviation for each group. Student T test was used for statistical analysis and P value was calculated. Correlation coefficient of serum, saliva and cell lysates of HSP 60 in periodontal health and disease was analysed using Pearson's correlation. $P < 0.05$ was considered to be statistically significant at the 5% level.

PATIENT GROUPS

GROUP A HEALTHY GINGIVA



GROUP B PERIODONTITIS



ARMAMENTARIUM



PROCESSING

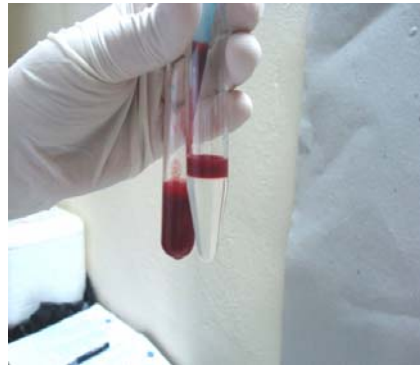
Vacutainer



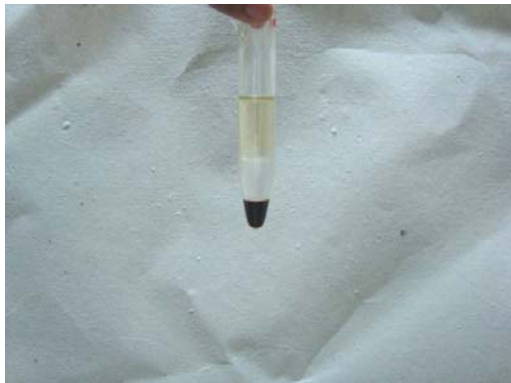
PBS + Blood



Layering



Buffy coat



cell sediment

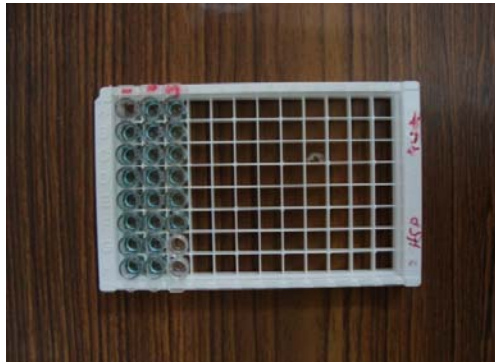


EQUIPMENTS

Centrifuge



Microplate



Automatic washer



vortex mixer



ELISA READER



REAGENTS

Histopaque-1077



HSP 60 Elisa kit



Sample collection

Blood sample



vacutainers



Saliva sample



**Sterile container for saliva
collection**



EVALUATION OF CIRCULATORY AND SALIVARY

RAGAS DENTAL COLLEGE AND HOSPITAL, CHENNAI.

DEPT OF PERIODONTICS

PATIENT PROFORMA

1. NAME :
2. AGE/SEX :
3. OCCUPATION :
4. ADDRESS :
5. PHONE :
6. CHIEF COMPLAINT :
7. PAST DENTAL HISTORY :
8. MEDICAL HISTORY :
9. PERIODONTAL EXAMINATION :

Gingiva

- a. Colour
- b. Contour
- c. Consistency
- d. Position

e. Surface texture

Recession:

Furcation Involvement:

Mobility:

Plaque Index

[illegible]

Bleeding Scores

[illegible]

PERIODONTAL PROBING DEPTH & CLINICAL ATTACHMENT LEVEL

[illegible][illegible]

BIOFILM GINGIVAL INTERFACE INDEX:

OTHER INVESTIGATIONS:

RADIOGRAPHIC INVESTIGATION:

IOPA:

OPG:

BLOOD INVESTIGATION:

1. Total Count:
2. Differential Count:
3. Bleeding Time:
4. Clotting Time:
5. Haemoglobin:
6. Random Blood Sugar:

DIAGNOSIS:

TREATMENT:

STATEMENT OF INFORMED CONSENT

Patient name:

Age/Sex:

I have been explained about the nature and purpose of the study in which I have been asked to participate. I understand that, I am free to withdraw my consent and discontinue at any Time without prejudice to me or effect on my treatment.

I have been given the opportunity to ask questions about the procedure. I have also Given consent for photographs, blood and saliva samples to be taken for the study purpose. I have fully agreed to participate in this study.

I hereby give my consent to be included in **“EVALUATION OF CIRCULATORY AND SALIVARY LEVELS OF HEAT SHOCK PROTEIN 60 IN PERIODONTAL HEALTH AND DISEASE”**

Station:

Date:

Signature of the patient:

Signature of the HOD :

RESULTS

The present study assessed the circulatory and salivary levels of HSP 60 in patients with periodontal health and disease by sandwich ELISA method.

45 peripheral blood samples and saliva samples were collected from the 2 groups. Group A or group B (22 health and 23 disease). The blood samples were processed. Serum and cell lysates were prepared to detect HSP 60 between the two groups

Absorbance was measured at 450nm wavelength in terms of ng/dl, and the results were obtained.

HS CRP (table 1)

The mean hs CRP level in periodontal disease was 2.25 ng/dl whereas the mean hs CRP in periodontal health was 1.16 ng/dl. There was a significant increase in the periodontal disease when compared to health at $p < 0.05$ (0.024).

Circulatory HSP 60 (table 2)

The mean circulatory HSP 60 levels were obtained by adding the mean cell lysate HSP levels and the mean serum HSP 60 levels. The mean circulatory HSP 60 level (total serum & cell lysate) in periodontal disease was 94.67 ng/dl whereas the mean value in periodontal health was 59.05 ng/dl.

There was a significant increase in the periodontal disease when compared to health at $p < 0.05$ (0.038)

When the circulating HSP60 levels were examined individually as cell lysate and serum levels, the results obtained were as follows:

HSP 60 Cell lysates (table 3)

The mean cell lysate level of HSP 60 in periodontal disease was 80.89 ng/dl whereas the mean cell lysate level of HSP 60 in periodontal health was 53.54 ng/dl. There was a significant increase in the periodontal disease when compared to health at $p < 0.05$ (0.044)

HSP 60 Serum (table 4)

The mean serum HSP 60 level in periodontal disease was 20.19 ng/dl whereas the mean serum HSP 60 level in periodontal health was 15.68 ng/dl. There was no significant increase in the periodontal disease when compared to health at $p > 0.05$ (0.488)

Correlation between circulatory HSP 60 in hs CRP in periodontal disease

(table: 6)

There was a statistically significant correlation between circulatory HSP 60 and hs CRP(57%) at $p < 0.05$.

HSP 60 Saliva (table 4)

The mean salivary level of HSP 60 in periodontal disease was 30.15 ng/dl whereas the mean salivary level of HSP 60 in periodontal health was 26.05 ng/dl. There was no significant increase in the periodontal disease when compared to health at $p > 0.05$ (0.628)

Correlation coefficient for HSP 60 levels in serum, cell lysates and saliva of periodontally healthy patients (table: 5)

There was no statistically significant correlation between the circulating HSP 60, salivary HSP 60 and hs CRP. Even when examined individually, there was no significant correlation between either serum HSP 60 or cell lysate HSP 60 and salivary HSP 60.

Correlation between salivary HSP 60 and circulatory HSP 60 and hs CRP in periodontal disease (table: 6)

There was no significant correlation between salivary HSP 60 and circulating HSP 60 (either total or individual). There was also no significant correlation between salivary HSP 60 and hs CRP.

Table 1

hs CRP levels in serum

Group	Number of cases	Mean	Standard deviation	P value
Health	22	1.16	0.033	0.024 [*]
Disease	23	2.25	0.29	

Table 2

Total circulatory HSP 60 levels in health and disease.

Group	Number of cases	Mean	Standard deviation	P value
Health	22	59.05	35.20	0.038 [*]
Disease	23	94.67	36.74	

Table 3

HSP 60 levels in cell lysates

Group	Number of cases	Mean	Standard deviation	P value
Health	22	53.54	30.52	0.044 [*]
Disease	23	80.89	49.46	

Table 4

HSP 60 levels in saliva and serum

Group		Mean	Standard deviation	P value
Serum	Health	15.68	10.02	0.488
	Disease	20.19	16.57	
Saliva	Health	26.04	14.36	0.628
	Disease	30.15	24.34	

Table 5

Pearson's correlation for total circulatory levels and hsCRP & HSP 60 levels in serum, cell lysates and saliva of periodontal health.

		Correlation coefficient	P value
Total circulatory levels	hs CRP	13%	0.754
Serum	Cell lysate	70%	0.995
Cell lysate	Saliva	10%	0.419
Saliva	Serum	3%	0.558

Table 6

Pearson's correlation for total circulatory levels and hsCRP & HSP 60 levels in serum, cell lysates and saliva of periodontal disease.

		Correlation coefficient	P value
Total circulatory levels	hs CRP	57%	0.052*
Serum	Cell lysate	15%	0.967
Cell lysate	Saliva	11%	0.280
Saliva	Serum	5%	0.347

Figure 1

Comparison of circulatory HSP 60 in periodontal health and disease

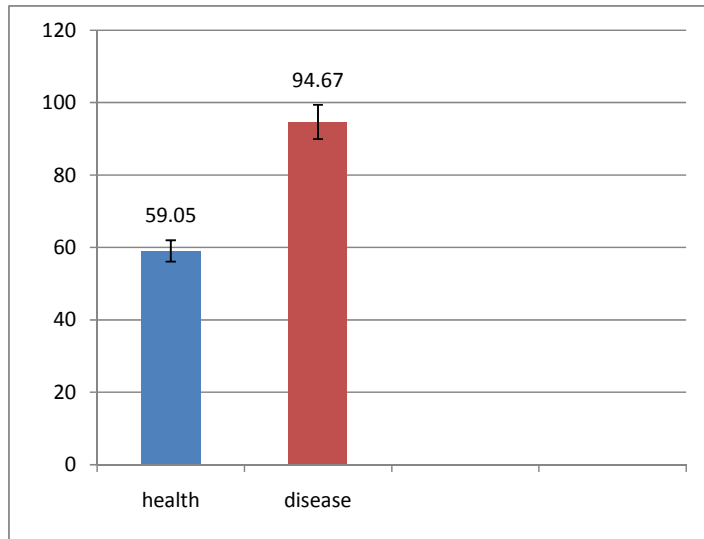


Figure 2

Comparison of serum HSP 60 in periodontal health and disease

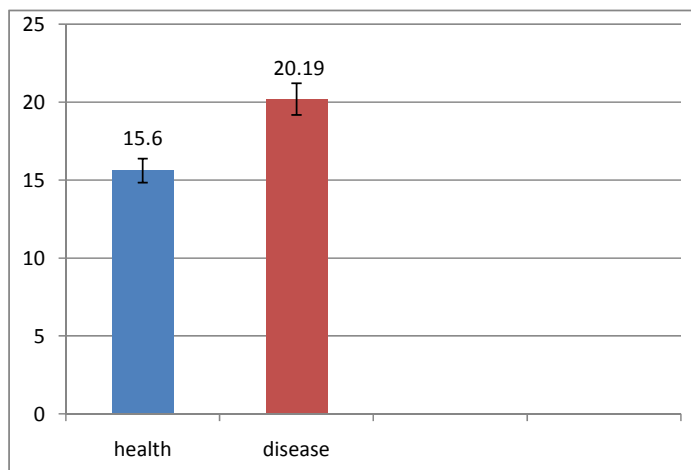


Figure 3

Comparison of cell lysate HSP 60 in periodontal health and disease

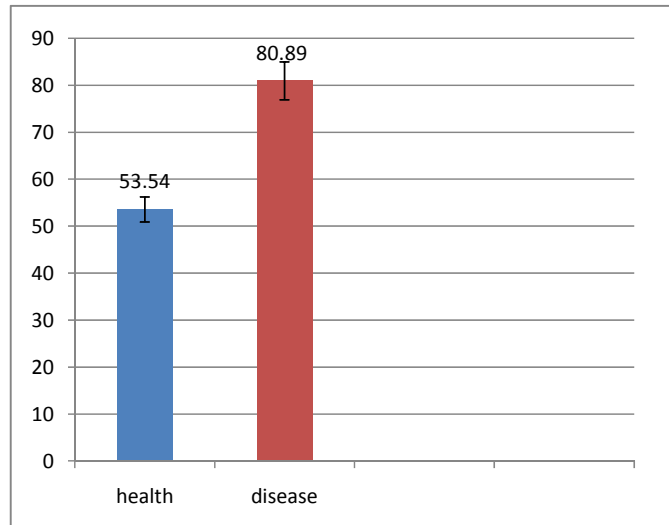
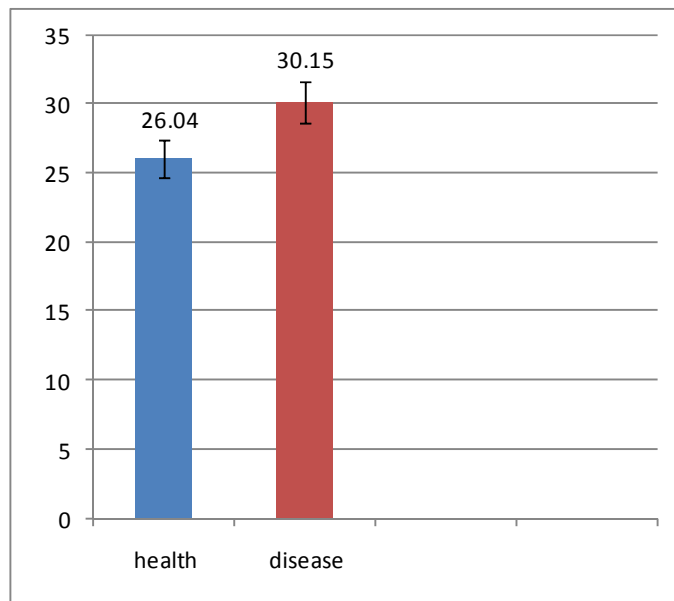


Figure 4

Comparison of salivary HSP 60 in periodontal health and disease



DISCUSSION

Although periodontopathic bacteria are the primary etiological agents in periodontal disease, the ultimate determinant of disease progression and clinical outcome is the host's immune response. The immune response is typically activated through exposure to bacterial antigens with specific Pathogen associated molecular patterns(PAMPs) such as LPS , fimbriae, & Outer membranous protein, etc.²³ In addition to these PAMPs, self antigens have also been reported to be involved in host inflammatory / immune response.^{75,81}

Among the most studied self antigen in periodontal disease is the heat shock protein, especially HSP 60. HSP 60 has been reported to be significantly elevated in gingival tissues affected by periodontal disease, but, there is no conclusive evidence linking it to disease activity. HSP's are typically regarded as intracellular proteins but free serum HSP 60 has been reportedly identified and linked to systemic inflammation and cardiovascular disease.^{10,30,57,33} The source of this free circulating HSP 60 remains a controversy. This study was undertaken to investigate if periodontal disease could contribute to the circulating HSP 60 levels in a manner similar to that of circulating proinflammatory cytokines.

Peripheral blood was collected from the antecubital fossa, and lymphocytes were separated from the peripheral blood using Histopaque-1077. Cell lysates were prepared using the extraction reagent provided in the heat shock protein 60 ELISA kit following standard protocol, as reported by other studies.^{3,60,49,}

Saliva was collected by the method, reported by *Navazesh et al (2008)*⁴³. Care was taken to ensure that, saliva was collected between 9-11 AM to avoid variation due to circadian periodicity that has been shown to affect salivary analytes.⁷¹

The ELISA method was used to evaluate HSP 60 in preference to immunoblotting as it is versatile, accurate, reproducible, and economical. Previous studies done by *Tabeta K et al (2001)*⁶⁸, *Watanabe S et al (2003)*⁷⁶ and *Handley HH et al (1995)*²⁴ provide evidence that supports the use of this method.

In our study, patients were divided only into health and periodontitis groups, a gingivitis group was not included. Previous studies have reported that gingivitis occurs as a non specific local inflammatory response to plaque & does not seem to produce a significant systemic inflammatory response.⁸⁰

Patients in the periodontitis groups were not sub classified into mild, moderate or severe groups for the following reasons

- 1) Previous results on the systemic inflammatory effects of periodontal disease have been inconclusive in detecting significant differences between mild, moderate and severe periodontitis. A paucity in the number of well controlled, prospective, longitudinal trials could be one reason for this lack of evidence.
- 2) We have used the BGI index in this study for clinical categorisation of chronic periodontitis.

The BGI proposed by *Barros and Offenbacher*, has been suggested to be dynamic and therefore a sensitive predictor of the systemic effects of periodontal disease. As the events occurring at the biofilm gingival interface demonstrably influence the inflammatory course, this index is thought to be a more reliable predictor of systemic inflammation. Only the BGI Deep lesion (BGI-DL) category was included due to practical difficulties in including an adequate sample of all groups for investigative procedures. Further, previous studies have reported that, systemic effects of periodontal disease were associated most significantly with the BGI-DL.⁴⁸

The result of our study indicate that, there was a significant increase in hs CRP levels in periodontal disease when compared to health. These results are in consistent with the reports of *Renvert, Nakajima et al.* The advantage of using hs CRP over conventional CRP is that it is more sensitive and there by provides a closer correlation to the inflammatory status.^{47,45} These results

seem to add on to the existing body of literature that suggests that periodontal disease is capable of evoking a low grade chronic systemic inflammation. There was no significant difference between the hs CRP levels and BGI MB and SB groups but the sample size of individual subgroup was too small for meaningful analysis. None of the patients included in our study belonged to the BGI-DL/LB category. A larger sample size could have provided predictive information about the reliability of the BGI index in populations with poor oral hygiene where gingival bleeding is very common. While these results cannot be compared directly, **Barros and co workers** have reported somewhat similar results in their study relating to HbA1c levels in diabetes mellitus samples.

The results of our study indicate that there was a significant increase in circulating HSP 60 in periodontal disease when compared to health. These results are in conformity with previous studies of **Ukei et al (2002)⁷³**, **Yamazaki et al (2002)⁸⁰**.

In order to further clarify its role in pathogenesis, cell lysate and serum HSP 60 levels were examined individually. The cell lysate HSP 60 levels were consistently higher when compared to serum in both health and disease groups. These results suggest that the primary source of circulatory HSP 60 was intracellular, present in the lymphocytes, rather than free in serum.

In periodontal disease, there was a significant elevation in cell lysate HSP 60 levels, while the serum levels showed no significant difference when compared to health. Although periodontal disease has been reported to result in a systemic spill over of proinflammatory cytokines such as IL-1, IL-6, TNF α etc,⁴⁷ other host derived products have not been extensively documented. The fact that periodontal disease was associated with an upregulation of cell lysate HSP 60 & not free serum HSP 60 suggests that this has not resulted from a mere systemic spill over. Whether this lymphocyte reaction was in response to HSP 60 1) of *Porphyromonas gingivalis* in gingival tissues or 2) *Porphyromonas gingivalis* in circulation or 3) in human fibroblasts, could not be determined. Previous evidence suggests the plausibility of any or all of the above.

Systemic cellular responses to locally generated antigens have been previously described in the form of hyper responsive monocytes.³² The ability of *Porphyromonas gingivalis* to enter circulation and colonize tissues other than gingiva has been well documented.⁸⁴ The structural homology between HSP 60 of *Porphyromonas gingivalis* with an epitope MQFDRGYISP and HumHSP 60 with an epitope MKFDRGYISP makes the third possibility distinctly plausible.⁶⁹ Further strength for this hypothesis may be gained from the generation of HSP 60 specific $\gamma \delta$ T cells that are normally involved in generation of self antigens.¹⁰

Similar to the results obtained with hs CRP, there was no correlation between circulatory HSP 60 levels, and BGI (MB and SB). The reason for this lack of association is not immediately apparent but the small sample size of the subgroup could have influenced the results. It may also be speculated that these self antigens are involved in early phases of established periodontitis and their further effects could probably be related to activation of the immune response.

Regardless of the pathogenic mechanism involved in the HSP 60 upregulation in periodontal disease; it is significant that it correlates positively with the systemic inflammatory status as indicated by hs CRP. The mechanisms through which HSP 60 could induce systemic inflammation have been well documented. In addition to activating Natural Killer Cells and $\gamma \delta$ T cells,¹⁰; these proteins can result in increase of proinflammatory cytokines like TNF α .⁷³ Both HSP 60 and hs CRP have been shown to be independently associated with cardiovascular disease.^{10,30,47,57,33} Therefore, upregulation of both circulating HSP 60 and hs CRP in periodontal disease could have important implications.

The association between periodontal disease and cardiovascular disease has gained enormous attention in recent years. Several investigators have found a strong association between the two diseases to the extent that a joint consensus report between the association of American cardiologists and

the periodontologists has addressed this issue.¹⁹ A number of pathogenic mechanisms have been suggested through which periodontal disease may affect cardiovascular disease.^{10,30,57,33}

Our study indicates that HSP 60 could be considered another potential candidate through which periodontal disease influences the systemic inflammatory state and thereby cardiovascular disease. The role of inflammation in the development of atheromatous plaques is now well established.⁷⁹

There has been a resurgent interest in salivary diagnostics in the last few years. Recent evidence demonstrated its ability to predict not only local inflammatory conditions such as chronic periodontal disease but also systemic conditions such as Cardiovascular disease, Breast cancer etc.¹ Our results suggests that salivary HSP 60 levels exhibited no significant difference between periodontal health and disease. There is scant previous literature with which we can compare these levels. Previous studies on HSP are related to HSP 70 levels and have focused largely on salivary gland pathology. Saliva has been reported to be a second hand marker of periodontal disease as most of its constituents have gained entry from GCF.³⁵ The lack of association between salivary HSP 60 levels and periodontal disease is probably due to its etiopathogenesis.

The role of HSP 60 in the etiopathogenesis of periodontitis may be described as follows:

Molecular mimicry with the HSP 60 of *Porphyromonas gingivalis* results in T cell activation against both the exogenous antigen as well the gingival fibroblasts. This leads to generation of T cell lines that may be destructive to the host tissue and result in excessive production of proinflammatory cytokines such as TNF α .⁷³

In other words; the pathogenic responses are restricted to immune complex activation against intracellular antigens (HSP 60 - either *Porphyromonas gingivalis* or gingival fibroblast) that are confined to the gingival tissue. Consequently; there is only a minimal presence of these proteins in inflammatory exudates and therefore, saliva. We hypothesize that due to these reasons; the salivary HSP 60 levels do not reflect periodontal disease activity.

Salivary HSP 60 did not correlate with the circulating HSP 60 levels or hs CRP. The cell lysate HSP 60 levels indicate that the predominant systemic response to HSP 60 is cellular in nature. As saliva is generally thought to be reflective of the plasma or serum constituents; there was no significant correlation between salivary and circulating HSP 60. Consequently, it did not correlate with hs CRP either. This data must not be interpreted to mean that saliva cannot be used as a diagnostic tool to evaluate systemic inflammation;

only that salivary HSP 60 is a poor marker of both periodontal disease and its systemic inflammatory response.

There are several limitations in this study including:

1. A small sample size which may have affected the statistical analysis.
2. Its cross sectional nature; longitudinal studies are better suited to study the episodic nature of periodontal disease & its biomarkers.
3. Immunolocalization of HSP 60 in gingival tissues could have perhaps given a more complete picture.

Within the limitations of this study; the significant association between periodontal disease, circulating HSP 60 and hs CRP indicates a strong possibility of the involvement of these self antigens in systemic inflammation. These results certainly warrant a more detailed investigation which might help clarify the role of these proteins in periodontal disease.

SUMMARY AND CONCLUSION

The aim of the present study was to estimate the circulatory and salivary levels of HSP 60 in periodontal health and disease and to assess whether they correlate with each other and with the systemic inflammatory marker, hs CRP. 45 patients who attended the out patient Department of periodontology, Ragas Dental College and Hospitals, Chennai were enrolled in the study. Patients were divided in to two groups based on their periodontal health status- 22 Healthy patients, 23 Periodontitis patients.

Peripheral blood and saliva was collected prior to phase I periodontal therapy from these patients and processed for ELISA analysis. Processing for ELISA involved the preparation of cell lysates and serum from peripheral blood. Cell lysates were prepared by adding the extraction reagent provided in the HSP 60 ELISA kit to the lymphocytes separated from the peripheral blood. Statistical analysis was done using student t test and Pearson's correlation

From the results of this study, we may conclude that

- 1) Periodontal disease results in an upregulation of circulating HSP 60 levels and the systemic inflammatory marker-hs CRP, both of which correlate significantly with each other.
- 2) Salivary HSP 60 has limited value as a biomarker of periodontal disease. Further, it correlates poorly with circulating HSP 60 levels and hs CRP and is therefore not an ideal surrogate marker of systemic inflammation.

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